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Functional residues at the active site of horse liver phosphopantothenoylcysteine decarboxylase

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Horse liver phosphopantothenoylcysteine decarboxylase (EC 4.1.1.36) is rapidly inactivated by *N*-acetoacetylation with diketene following a pseudo-first-order kinetics: the presence of substrate quantitatively protects against this inactivation. Histidine photo-oxidation with methylene blue or rose bengal brings about the total loss of activity. These results indicate the presence of functional lysyl and histidyl groups at the active site of the enzyme. The substrate sulphydryl group is essential for enzyme activity. Enzymatic decarboxylation is proposed to result from a combined action of the keto group of the enzyme-bound pyruvate protonated by an essential histidine and a protonated amino group of a lysine.

Phosphopantothenoylcysteine decarboxylase; Pyruvate; Functional residue; (Horse liver)

1. INTRODUCTION

Phosphopantothenoylcysteine decarboxylase (EC 4.1.1.36) catalyzes the decarboxylation of 4′-phosphopantothenoyl-L-cysteine to 4′-phosphopantetheine. The reaction is a key step in coenzyme A biosynthesis [1] and represents in mammals the only known mode to decarboxylate cysteine [2]. The enzyme purified from bacterial sources [3] and rat and horse liver [4,5] does not contain PLP but pyruvate [6] linked to the enzyme as an ester rather than as an amide [7].

Few models have been proposed to describe the catalytic mechanism of the bacterial and rat liver enzymes [3,8]. To elucidate the molecular basis for horse liver PPCDC catalytic activity we initiated chemical modifications of the enzyme amino acid residues and we tested as potential substrates PPC with a modified sulphydryl group.

These experiments indicate that the enzyme has functional lysyl and histidyl groups. Moreover the substrate sulphydryl group is strictly required for enzyme activity.

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Abbreviations: PPCDC, 4′-phosphopantothenoyl-L-cysteine decarboxylase; PLP, pyridoxal-5′-phosphate; PPC, 4′-phosphopantothenoyl-L-cysteine; Hepes, *N*-hydroxyethylpiperazine-*N*-2-ethane sulphonate; pMB, *p*-chloromercuribenzoate; TNBS, 2,4,6-trinitrobenzenesulfonic acid; DPC, diethylpyrocarbonate

2. MATERIALS AND METHODS

PPCDC was purified as described in [5]. Enzyme assays were carried out by measuring the $^{14}CO_2$ released from labeled PPC [9]. Various reagents for chemical modifications were used at the concentrations indicated in the text; modifications were usually carried out at 25°C in 50 mM Hepes buffer, pH 7.5 or 8.5, with 0.25 mg/ml of protein and, when used, 0.5 M urea, a concentration [9] which favours access to the enzyme active site of either the substrate or the modifier. To terminate the reactions, aliquots were diluted at least 20-fold in the assay mixture for PPCDC activity. Residual reagents did not interfere with activity assays. Histidine photochemical oxidation of 1.0 mg/ml enzyme solution in 50 mM phosphate buffer, pH 7.6, was performed with methylene blue or rose bengal, 0.01% (w/v), at 15°C by a 150 W light source, placed at 10 cm from the incubation mixture. After 10 min the dye was removed by gel filtration on Biogel P6. The dyes had no effect on enzyme samples shielded from irradiation. To exclude the involvement of the SH groups in the inactivation exerted by the dye, the enzyme was treated with 1 mM pMB prior to the addition of the reagent and the succeeding photo-oxidation. The activity of the

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sample treated with both the pMB and the dye measured after mercaptoethanol addition was identical with that found for the enzyme treated with the dye alone (table 1).

To irreversibly modify the substrate sulphydryl group, radiolabeled PPC was first reduced by a 25-fold excess of cysteine under a nitrogen stream at pH 8.5 Hepes, for 30 min. The cysteine was then removed by Dowex 50 WX4, H^+ and the solution concentrated by rotary evaporation under reduced pressure at 30°C. Substrate sulphydryl groups were titrated with Ellman's reagent [10].

Reduced PPC was supplemented with bromopyruvic acid in a 1:2 ratio, in 1 M phosphate buffer, pH 8.0, at room temperature; the pH was maintained at 8.0 (paper) with diluted NaOH. The reaction was stopped after 15 min by adding 2-mercaptoethanol stoichiometric to bromopyruvate. Reduced PPC was methylated by adding a 20-fold excess of iodomethane. After the reaction was completed, the excess of iodomethane was removed by rotary evaporation under reduced pressure. Methylation of substrate sulphydryl group was confirmed by NMR detection of the *S*-methyl group signal (singlet at 2.1 δ relative to DSS as internal standard in D_2O). Both modified substrates were tested for PPCDC activity immediately after preparation. All reagents were from Sigma.

3. RESULTS

Incubation of PPCDC with increasing diketene concentrations in 50 mM Hepes buffer, pH 8.5, results in a time-dependent loss of enzyme activity (fig.1). The inactivation process goes to completion following a pseudo-first-order kinetics. The slope of the double logarithmic plot of the reciprocal of the half-times of inactivation against diketene concentration [11] yields a reaction order of 0.9 with respect to diketene, indicating that interaction of at least one molecule of reagent with an enzyme site leads to inactivation. The substrate protects against diketene inactivation (fig.2) and the decrease in the rate of inactivation process

Table 1

Enzyme photooxidation

Reagent	Conc. (w/v)	pH	Time (min)	Activity ($V_i/V_c \times 100$)
None	–	7.6	10	100
Rose bengal	0.01%	7.6	10	0
Rose bengal (shielded)	0.01%	7.6	10	100
Rose bengal + 1 mM PMB	0.01%	7.6	10	0
Rose bengal + 1 mM PMB (shielded)	0.01%	7.6	10	100

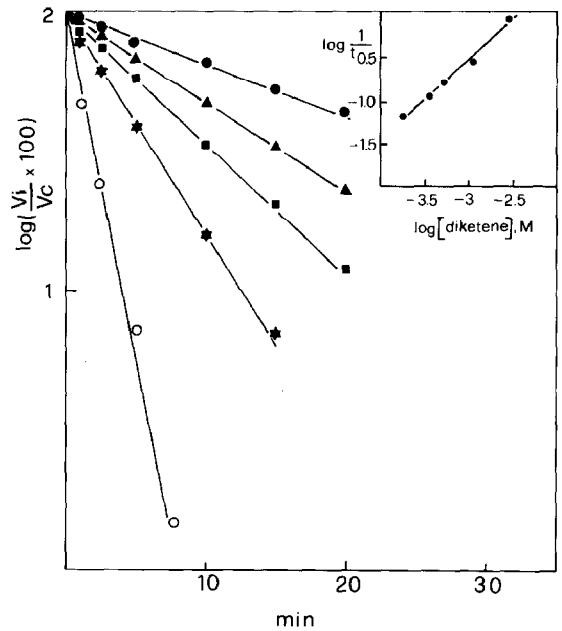

Fig.1. Time course of PPCDC inactivation by diketene. PPCDC was incubated in 50 mM Hepes buffer, pH 8.5, with 0.18 (●—●), 0.37 (▲—▲), 0.55 (■—■), 1.15 (★—★) and 3.00 mM (○—○) diketene. At the indicated times, samples were diluted 20-fold and assayed for activity reported as log of the ratio of that of the modified enzyme, V_i, and the unmodified control, V_c, × 100. (Inset) Order of PPCDC inactivation with respect to diketene concentration. The data of the time course are replotted in the form of log $1/t_{0.5}$ (min) as a function of log [diketene]. The slope of the line yields a value of 0.9.

depends on PPC concentration. The pseudo-first-order rate constants for inactivation determined at PPC initial concentrations ranging from 0.62 to 2.50 mM were used to determine a 1.1 mM apparent dissociation constant for the enzyme-PPC complex by use of the equation [12]:

$$V_a/V_o = k_2/k_1 + K_d\frac{(1 - V_a/V_o)}{[PPC]} \quad (1)$$

where V_a and V_o represent respectively the pseudo-first-order rate constants for inactivation in the presence and absence of PPC; k_2 and k_1 are the fractional order rate constants for inactivation of free enzyme and enzyme-PPC complex (reaction sequences 2 and 4); K_d is the dissociation constant for E-PPC (reaction sequence 3):

$$E + 0.9\ \text{diketene} \xrightarrow{k_1} E\ (0.9\ \text{diketene}) \quad (2)$$

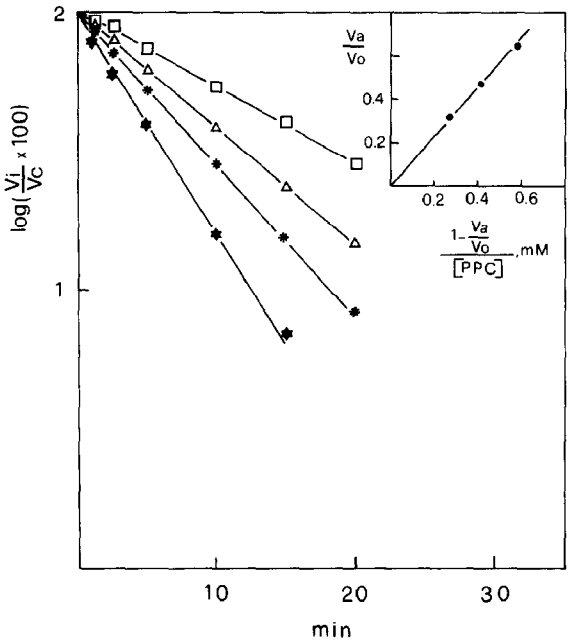

Fig.2. Protection by substrate of PPCDC inactivation by diketene. The enzyme was modified with 1.15 mM diketene as in fig.1 (★—★) and protected with initial PPC concentrations of 0.62 (*—*), 1.25 (△—△) and 2.50 mM (□—□). (Inset) Determination of apparent dissociation constant for the E-PPC complex. Data are plotted according to eqn 1 in the text. V_a and V_o are the reciprocals of the inactivation half-times (min) for protected and unprotected enzyme, respectively.

$$E + PPC \overset{K_d}{\rightleftharpoons} E\text{-}PPC \quad (3)$$

$$E\text{-}PPC + 0.9 \text{ diketene} \xrightarrow{k_2} E\text{-}PPC\ (0.9 \text{ diketene}) \quad (4)$$

The inset of fig.2 shows a plot of V_a/V_o versus $(1 - V_a/V_o)/[PPC]$. A straight line passing through the origin is obtained demonstrating that E-PPC complex is completely protected against diketene inactivation, i.e., k_2 is very small compared with k_1. The slope of the line yields an apparent dissociation constant, K_d, for the E-PPC complex of 1.10 mM close to the K_m value for PPC of 1.43 mM [9]. The involvement of at least one lysine residue in the catalytic activity of PPCDC was confirmed by the inhibitions exerted either by PLP or by TNBS, both prevented by the presence of the substrate (table 2).

Butanedione, 10 mM, in 50 mM borate buffer, pH 8, for 90 min yields a 20% inactivation, indicating that arginyl residue(s) are unessentially involved in the enzyme function. The fact that the presence of substrate in the K_m range does not protect against the reagent rules out the possible catalytic role of arginyl residue(s) (table 2).

Table 2

Modification of PPCDC by various reagents

Reagent	Conc. (mM)	pH	Time (min)	Activity ($V_i/V_c \times 100$)
None				100
Urea	500	7.5	60	100
Butanedione[a]	10	8.0	90	80
Butanedione[a] + 2 mM PPC	10	8.0	90	80
DPC	2	7.5	60	70
DPC + 500 mM urea	2	7.5	60	50
DPC + 2 mM PPC	2	7.5	60	85
Acetylimidazole	5	7.5	60	100
TNBS	1	8.5	10	0
TNBS + 2 mM PPC	1	8.5	10	50
PLP	5	8.5	15	30
PLP + 2 mM PPC	5	8.5	15	80

[a] Reaction was in 50 mM borate buffer. All other reactions were in 50 mM Hepes buffer

Over a period of 60 min (table 2) a 30% inactivation is brought about by 2 mM diethylpyrocarbonate at pH 7.5, increased to about 50% by addition of 0.5 M urea. The substrate protects against this inactivation. Deacylation with hydroxylamine has not been performed because of the high reactivity of PPCDC with carbonyl reagents [9].

Enzyme activity is unaffected by 5 mM acetylimidazole within 1 h at pH 7.5.

Remarkably, enzyme photo-oxidation in the presence of methylene blue or rose bengal (see details in section 2) causes the total loss of enzymatic activity completely prevented by shielding from irradiation. Photo-oxidation of enzyme SH groups was ruled out by experiments with pMB reported in table 1. To test the importance of the substrate sulphydryl group we synthesized the methyl and pyruvoyl derivative of PPC. Both are not substrates for PPCDC over the concentration range from 10^{-4} to 10^{-2} M indicating that the substrate sulphydryl group is critically involved in substrate binding.

4. DISCUSSION

PPCDC from horse liver has essential lysyl and histidyl groups. Lysyl residues are known to react selectively with diketene which irreversibly modifies PPCDC within 20 min of incubation. The protection from diketene inactivation by substrate, points to a central role of at least one lysyl residue in the catalytic activity of PPCDC. The presence of essential lysyl residue(s) was confirmed by the inhibition obtained either by PLP (previously observed [9]) or by TNBS both removed by the substrate.

Pantothenoylcysteine is not a substrate for the horse and rat liver enzyme [4,9], indicating that the phosphate group is essential for substrate binding. Our results indicate that a lysyl rather than an arginyl residue [13] binds the negatively charged phosphate group, since butanedione in borate buffer causes a modest loss in activity, unaffected by the presence of substrate.

The failure to inhibit the enzyme activity by acetylimidazole would preclude any importance for tyrosyl residues in PPCDC.

The histidyl residue(s) are essential for enzyme activity which is irreversibly lost by photochemical oxidation, but remarkably diethylpyrocarbonate does not yield the same results as the oxidative procedure, giving rise to only a modest inactivation. This result indicates that the catalytically important histidine is poorly accessible to the reagent, which however reaches the active site in the presence of 0.5 M urea (table 2). Further investigations on DPC inactivation was not performed since this reagent is active towards lysyl residues as well [14].

In the horse and bacterial enzyme mild base hydrolysis causes the release of pyruvate [3,6], suggesting that the pyruvoyl group is bound to the protein by an ester linkage [7], probably with a serine.

The importance of sulphydryl groups in PPCDC has been noted since our first report [9]. Irreversible modifiers of protein SH groups such as iodoacetamide and 4,4′-bis(dimethylaminodiphenylcarbinol) are inhibitors of PPCDC, demonstrating that the enzyme has SH groups involved in catalytic activity. The lack of decarboxylating activity towards the pyruvate adduct and the methyl derivative of PPC stress the role of the substrate sulphydryl group as an interacting site with the enzyme.

In the field of pyruvate containing enzymes, the only well established mechanism is that of histidine decarboxylase. In 1970 Recsei and Snell [15] demonstrated, by trapping the key intermediate, that decarboxylation proceeds via Schiff base formation, in analogy with PLP-dependent decarboxylation reactions.

In PPCDC the Schiff base formation has been excluded [8] since in this case the amino group of the cysteine moiety of the substrate is engaged in a usually very stable and unreactive amide linkage. Two different proposals have been therefore advanced recently.

The mechanism proposed by Aberhard et al. [8] in connection with the crucial finding that decarboxylation is stereospecific, requires the formation of a carbanion stabilized by a C–C interaction with the pyruvate keto group. In our opinion the formation of a C–C bond for a 'temporary' binding of the presumed carbanion resulting from carbon dioxide elimination seems unlikely.

To supply the appropriate stabilization for an analogous carbanion intermediate, Yang and Abeles [3] propose the involvement of the pyruvate keto group in the formation of a nitrogen ylide. It has been reported that sulphur and phosphorus ylides are strongly stabilized by resonance and other causes, whilst nitrogen ylides are stabilized only by field effect [16]. In our opinion the improvement of stability of the intermediate carbanion by the intervention of the nitrogen ylide seems too small to explain a so large kinetic effect like that required by a catalytic reaction.

In the light of our experimental results we present a mechanism which seems to satisfactorily account for the role of (i) the pyruvate; (ii) the amino acid residues essential for the catalytic activity; (iii) the stereochemical outcome of the decarboxylation [8].

As shown in fig.3 the substrate at the active site of the enzyme undergoes decarboxylation through the combined action of the keto group of pyruvate, protonated by an essential histidine, and the protonated amino group of the catalytic lysine.

The formation of intermediate I requires the nucleophilic attack of the substrate amide nitrogen on the keto group of the pyruvate followed by proton exchange and dehydration. Unlike amines

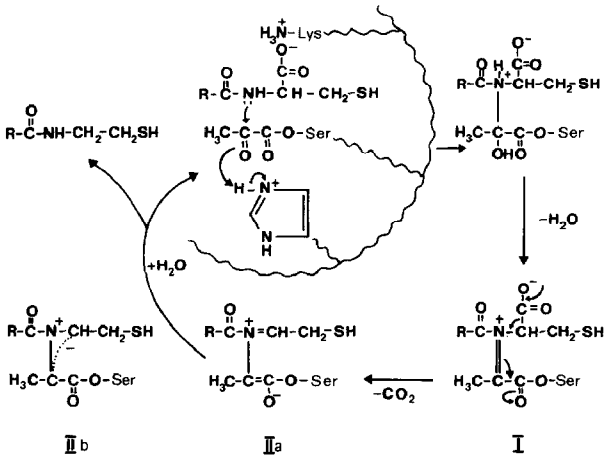

Fig.3. Proposed mechanism for the enzymatic decarboxylation of PPC.

which easily form Schiff bases, carboxamides are very weak nucleophiles and their reactivity towards carbonyl groups is generally very sluggish. However, successful condensations between carboxamides and particularly reactive carbonyl compounds [17] like pyruvic acid [18,19], even under mild conditions, have been reported. The acid-catalysed addition of the amide nitrogen to the pyruvic keto group should give an intermediate carbinolamide which gives rise in turn to the final products. Bicyclomycin [20] and several of its synthetic analogues can be considered outstanding examples of exceedingly stable cyclic carbinolamides where a carboxamide group adds to the keto group of the α-keto acid moiety of the molecule.

In the present mechanism protonation of the pyruvate keto group is efficiently carried out by the functional histidine. The intermediate I strictly resembles the protonated Schiff base proposed by Recsei and Snell for histidine decarboxylase [15]. Decarboxylation will therefore proceed in the same way and the catalytic histidine may intervene again to protonate the carbonyl oxygen of the ester pyruvate in order to enhance its electron withdrawal. Loss of CO_2 leads to the intermediate IIa which can now accept a proton from the protonated amino group of the catalytic lysine with complete retention of configuration.

Grigg and co-workers [21] have recently shown that 1,3-dipoles ($-\overset{|}{C}-\overset{|}{\underset{1+}{N}}-\overset{|}{C}-$) intervene in the decarboxylation of imines of amino acids and proposed that similar intermediates might be important in the biochemistry of pyridoxal. We suggest that this hypothesis may be extended to the pyruvate-dependent decarboxylases: thus the intermediate IIa may be regarded as a 1,3-dipole (IIb).

Appropriate trapping experiments with dipolarophiles are in progress in order to provide experimental evidence in supporting this hypothesis.

The present proposal seems particularly attractive since it assigns the same role to the pyruvate in pyruvate-dependent decarboxylases and allows the unification, at mechanistic levels, of both pyruvate and PLP-dependent decarboxylases.

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